

VEGF induces signalling and angiogenesis by directing VEGFR2 internalisation via macropinocytosis

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Figure S1

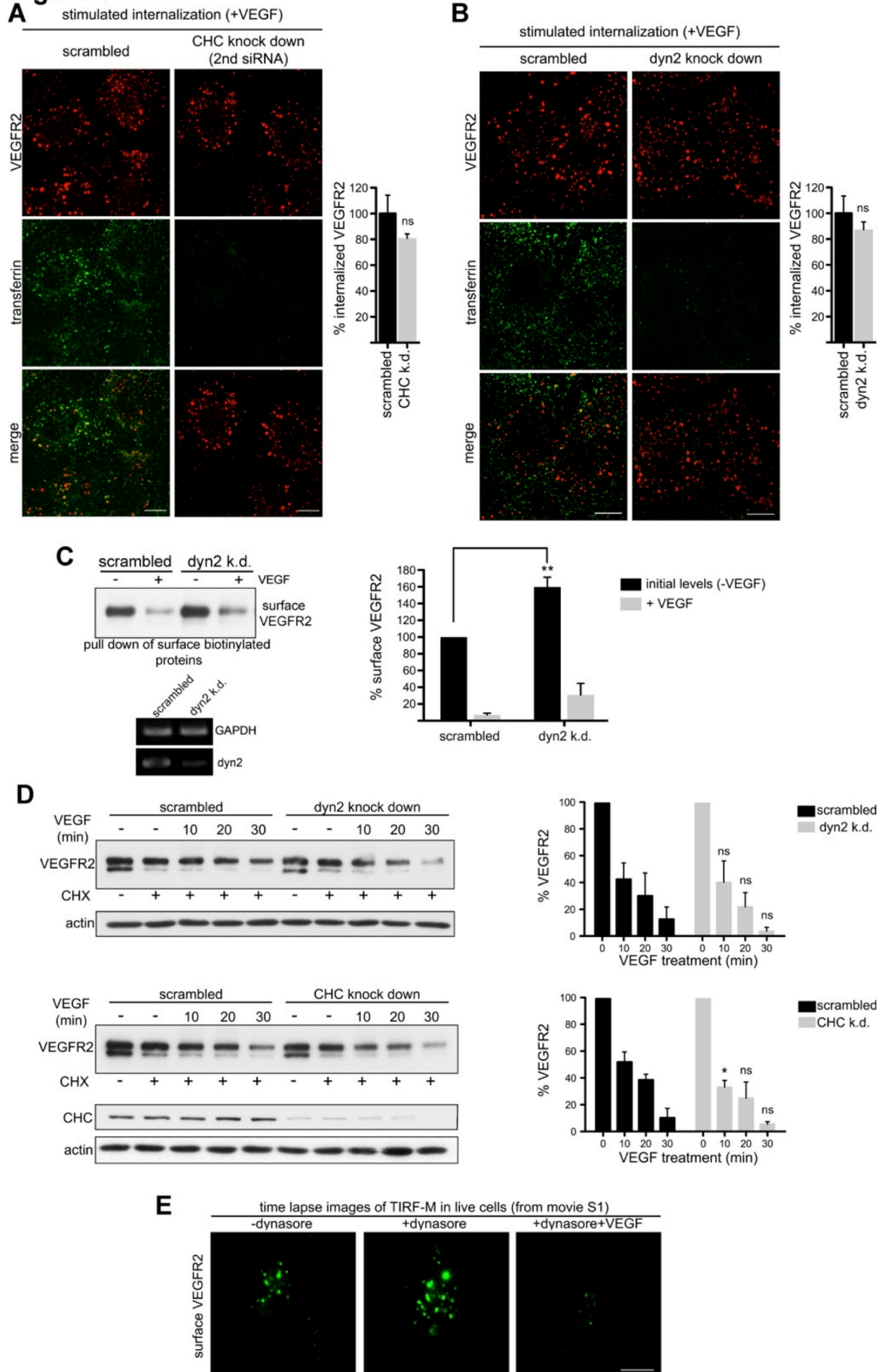


Figure S1. VEGF-induced Internalization of VEGFR2 is clathrin- and dynamin-independent.

HUVECs treated with siRNAs against CHC (A) or dynamin 2 (B) were incubated with a mouse anti-VEGFR2 extracellular domain antibody at 4⁰C, transferred to 37⁰C and the receptor was allowed to internalise for 15 min, in the presence of VEGF and FITC-transferrin. Prior to fixation, membrane bound antibodies and transferrin were removed by acid wash and the internalised receptor was revealed by fluorescently labeled secondary antibodies, using confocal microscopy. Inhibition of transferrin uptake verified the effective inhibition of clathrin- or dynamin-mediated endocytosis. Quantification of VEGFR2 internalisation is shown on the right of the immunofluorescence images (10 μ m scale bars). Data shown are representative of 3 independent experiments (n=6 microscopy fields of approximately 25 cells, mean \pm S.D., ***P<0.001, t-test).

(C) HUVECs treated with siRNAs against dynamin 2 were incubated with VEGF for 15 min, transferred to 4⁰C and surface proteins were labeled with cell impermeable biotin. Surface biotinylated proteins were pulled-down by streptavidin-beads and analysed by immunoblotting. Surface VEGFR2 was revealed using rabbit anti-VEGFR2 antibodies. Quantification of VEGFR2 internalisation is shown on the right of the immunoblots (n=3, mean \pm S.D., t-test).

(D) HUVECs treated with siRNAs against CHC or dynamin 2 were serum starved for 2h, incubated with 100 μ m cycloheximide for 30 min and stimulated with VEGF for the indicated time intervals. Quantification of VEGFR2 levels is shown on the right of the immunoblots (n=3, mean \pm S.D., ***P<0.001, two-way ANOVA, Bonferroni).

(E) HUVECs transiently expressing VEGFR2-mCherry were monitored by TIRF microscopy (see movie S1). Dynasore and VEGF were added sequentially to the cells. Images were taken at quiescence (steady state, left image), 30 min after addition of dynasore (middle image), and 15 min after the addition of VEGF (right image). Penetration depth was set to 150 nm. Note that addition of dynasore leads to an increase of the signal of VEGFR2 at the plasma membrane (due to inhibition of constitutive internalization), while the subsequent addition of VEGF results in progressive loss of the plasma membrane signal of VEGFR2 (due to VEGF-induced internalization in a dynamin-independent manner). Scale bars represent 10 μ m.

Figure S2

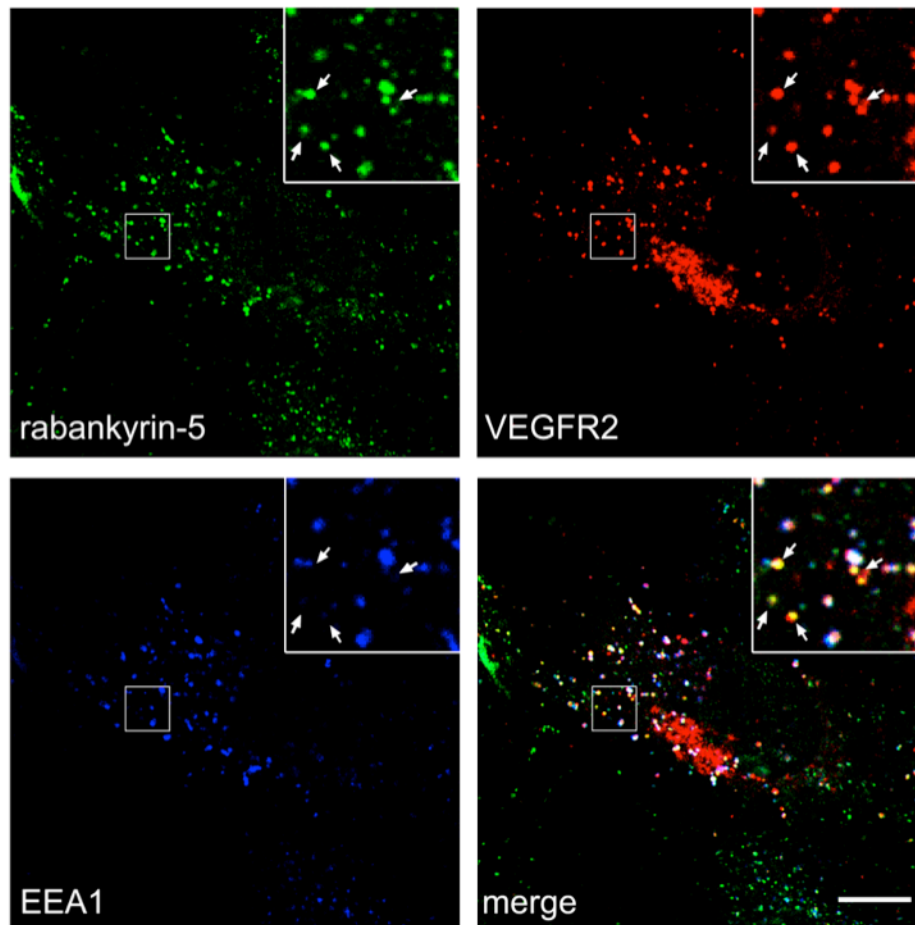


Figure S2. Immunofluorescence microscopy analysis of the distribution of VEGFR2 in EEA1- and Rabankyrin-5-positive endosomes.

HUVE cells were stimulated with VEGF for 15 min, fixed and processed for immunofluorescence analysis using goat anti-VEGFR2 extracellular domain antibodies. Endogenous EEA1 and Rabankyrin-5 were detected by anti-EEA1 and anti-Rabankyrin-5 antibodies. Scale bar represents 10 μ m.

Figure S3

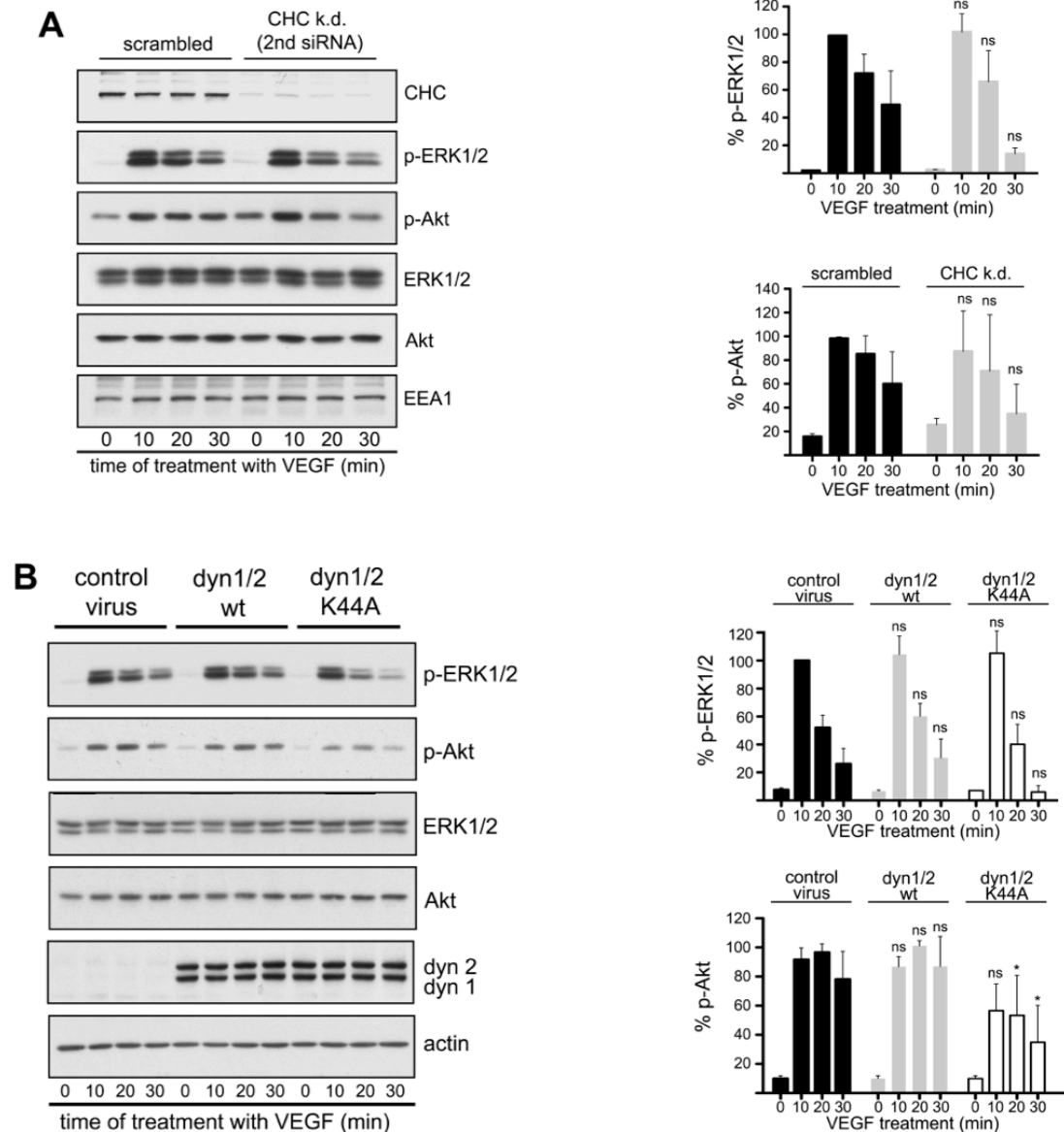


Figure S3. Clathrin- or dynamin-mediated endocytosis is redundant for VEGF signalling.

HUVECs transfected with siRNAs against clathrin heavy chain (**A**), or transduced with lentiviral vectors encoding dynamin (1 and 2) or dynamin K44A (1 and 2) (**B**), were stimulated with VEGF and subjected to immunoblotting analysis using antibodies against ERK1/2 and Akt (phosphorylated or total). Bar graphs on the right show quantification of the immunoblots ($n=3$, mean \pm S.D., $*P<0.05$, two-way ANOVA, Bonferroni).

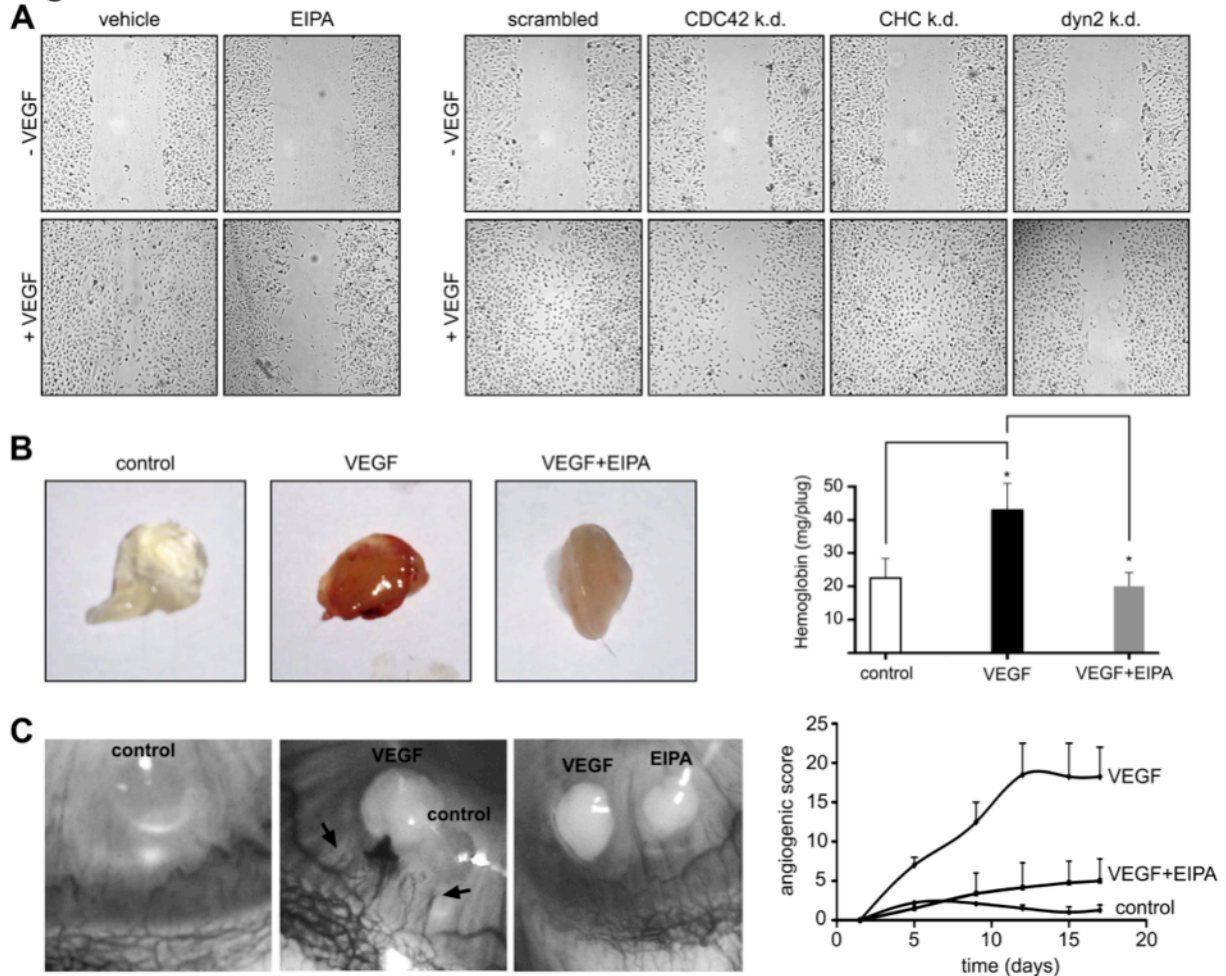
Figure S4

Figure S4. Macropinocytosis is critical for VEGF-induced migration of endothelial cells and *in vivo* angiogenesis.

(A) Representative images of the effect of EIPA, or the knockdown of CDC42, or CHC, or dynamin 2, on the VEGF-induced migration of HUVECs. Images of scratched areas were captured at 0h (*upper images*) and 14h post-stimulation of cells with VEGF (*lower images*).

(B) Representative pictures of Matrigel plugs at day 7 after subcutaneous implantation. Quantitative analysis of angiogenesis in the Matrigel plugs is shown on the right.

Haemoglobin content of plugs at day 7 is shown ($n \geq 4$, mean \pm S.D., * $P < 0.05$, one-way ANOVA, Dunnett).

(D) Representative images of rabbit corneal angiogenesis assays in response to empty pellet (left), VEGF + empty pellets (middle) or VEGF + EIPA pellets (right). Pellets are indicated by the corresponding labels inside the images. Arrows indicate newly formed vessels. Pictures show samples at day 7 post-implantation. Quantification of VEGF-induced vascularization (as angiogenesis score) over time is shown at the bottom (mean \pm S.E.M. of 3-4 implants, two-way ANOVA, Bonferroni). After day 5, the score of VEGF-induced angiogenesis is statistically different from the samples treated with empty pellet or VEGF+EIPA.

LEGENDS TO MOVIES

Movie S1. VEGF induces dynamin-independent internalisation of VEGFR2. (Related to Figure 1)

HUVECs transiently expressing VEGFR2-mCherry were monitored by TIRF microscopy. Dynasore and VEGF were added sequentially at the time points indicated in the movie. Penetration depth was set to 150 nm, images were acquired every 15 sec and reconstructed into a movie. Note that addition of dynasore (at t=00:06:29) leads to an increase of the signal of VEGFR2 at the plasma membrane (due to inhibition of constitutive internalisation), while the subsequent addition of VEGF (at t=00:54:24) results in progressive loss of the plasma membrane signal of VEGFR2 (due to VEGF-induced internalisation in a dynamin-independent manner).

Movie S2. VEGF induces membrane ruffling followed by internalisation of VEGFR2 into large vesicles. (Related to Figure 2)

HUVE cells transiently expressing GFP-actin and VEGFR2-mCherry were monitored by live cell time-lapse confocal microscopy in the presence of VEGF. Arrows indicate sites at the plasma membrane that undergo extensive ruffling (seen by the dynamics of GFP-actin) and give rise to enlarged VEGFR2-positive vesicles (seen by VEGFR2-mCherry). A magnified region of the plasma membrane is shown in the inset video at the top left corner of the movie. Plasma membrane ruffling starts 10 min following VEGF stimulation.